Subterranean Biology 45: 53–74 (2023)
doi: 10.3897/subtbiol.45.97105
https://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

First data on testate amoebae associated with the endemic cave bivalve *Congeria jalzici* Morton & Bilandžija, 2013 with a description of *Psammonobiotus dinarica* sp. nov.

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Academic editor: R. Mayén-Estrada | Received 3 November 2022 | Accepted 22 January 2023 | Published 21 February 2023

https://zoobank.org/3CEBADA2-26F6-4861-9ABF-746EFB65C85A

Citation: Baković N, Siemensma F, Puljas S, Baković R, Ozimec R, Ostojić A, Mesić Z (2023) First data on testate amoebae associated with the endemic cave bivalve *Congeria jalzici* Morton & Bilandžija, 2013 with a description of *Psammonobiotus dinarica* sp. nov. Subterranean Biology 45: 53–74. https://doi.org/10.3897/subtbiol.45.97105

Abstract

Testate amoebae are phylogenetically a very diverse group of eukaryotic microorganisms. They can be found in marine and freshwater habitats and in soil. Some of these single-celled organisms inhabit both surface and cave habitats, but their diversity in caves has barely been explored. Recent studies in the Dinaric region imply that testate amoebae in caves show a high diversity. The aim of this study was to identify the alpha diversity of testate amoebae in the Lika region (Dinaric karst, Croatia) and to compare the habitats of different caves based on testate amoebae assemblages. In eight caves we found more than 40 testate amoebae taxa, including a new testate amoeba species, *Psammonobiotus dinarica* **sp. nov.** The greatest diversity of testate amoebae was found in Markov ponor (27 taxa). The Bray-Curtis Similarity Index showed that testate amoebae assemblages in caves inhabited by the endemic and endangered cave bivalve *Congeria jalzici* (Markov ponor, Dankov ponor and Dražice ponor) differ from caves not inhabited by this species. This differentiation is attributed to the impact of the sinking Lika river, which occasionally completely submerges these caves, creating specific habitats for eukaryotic microorganisms. This study contributes to our understanding of the diversity, biogeography and ecology of testate amoebae in caves, as well as providing further insight into the conditions that sustain populations of *C. jalzici*.

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Keywords
cave flooding, cave heterogeneity, cave protists, *Centropyxis*, *Difflugia*, hygropetric, Markov ponor, *Microchlamys patella*, psammobiotic testate amoebae, sinkholes, unicellular cave organisms

Introduction

The Dinaric karst of the Western Balkans is world's most important subterranean biodiversity hotspot and is classified as a unique and rare ecosystem with numerous endemic species (Sket et al. 2004; Culver et al. 2006; Sket 2012). Biodiversity uniqueness has been recognized by a subterranean community that reflect adaptations to specific conditions and past intense geologic events that drove speciation and migration (Culver and Sket 2000).

Among the representatives of stigobionts as aquatic species in Europe, only one genus of bivalves is represented in caves. The Tertiary relict genus *Congeria* currently survives as three distinct species, namely *Congeria kusceri* Bole, 1962, *C. mulaomerovici* Morton & Bilandžija, 2013, and *C. jalzici* Morton & Bilandžija, 2013, with a highly fragmented distribution (Bilandžija et al. 2014; Jovanović Glavaš et al. 2017). All three species occur in only 15 caves in the Dinaric karst region (Jalžić 1998, 2001; Bilandžija et al. 2014; Jovanović Glavaš et al. 2017), but the rapid decline or disappearance of *Congeria* populations observed in recent decades underscores that much more needs to be done for the species conservation (Bilandžija et al. 2021).

Among the many ecological traits of *Congeria* (Morton and Puljas 2013; Puljas et al. 2014), the most interesting is adaptation to extreme habitat variations (presence of water and temperatures). Sinkholes with *Congeria* in the Lika region are completely or partially flooded only during high water events. These flooding events are partly controlled by the operation of the hydropower system on the Lika River. When these sinkholes are flooded, they provide an optimal habitat for *Congeria*, as it allows them to feed and reproduce. After the flooding of the Lika River waters subside, the slow decline of the water level in the sinkholes starts. Habitats that were once running water slowly turn into lake habitats and eventually the water is completely depleted. After that, the water on *Congeria* habitats is limited to seeping water that comes from the epikarst layer above the cave and wet walls. When *Congeria* habitat is converted to non-aquatic habitat, the most extreme conditions for the species begin. The importance of this habitat is usually discussed in the literature as an extreme part of the life cycle of *Congeria* (Jovanović Glavaš et al. 2017; Bilandžija et al. 2021), but there are no further data on the habitat itself. There are many active sinkholes in the Dinaric karst region and many caves with constantly flooded channels, but the distribution of *Congeria* species is limited to just a few localities. This implies that caves with *Congeria* hold some traits that are unique and differentiate them from other caves.

Despite the fact that *Congeria* species are under high threats both due to various human interventions (e.g. hydrotechnical projects) and climate change (Bilandžija et al. 2021), very little data is available on this species. Previous studies of *Congeria* spp. have included some data on species biology and population structure (Jalžić 1998, 2001; Morton et al. 1998; Morton and Puljas 2013; Bilandžija et al. 2014; Puljas et al. 2014; Jovanović Glavaš et al. 2017), evolutionary biology (Stepien et al. 2001; Bilandžija et al. 2014) and viruses in *Congeria* species (Scapolatiello et al. 2022). Relationships of *Congeria* and unicellular eukaryotic microorganisms (protists) have never been investigated. Only data on protists from caves with *Congeria* are findings of algae in the aphotic zone of Markov ponor, Dankov ponor and Dražice ponor, noted by Baković et al. (2022b). Their presence was attributed to flood waters of the Lika River. When discussing cave protists in the context of *Congeria*, we can only make some hypotheteses based on the general ecology of these groups of organisms. Some cave protists could compete with *Congeria* for food (e.g., organic particles, bacteria) (Hausmann et al. 2003) or even represent a compatible food source for *Congeria* (very small eukaryots) (Morton et al. 1998; Gosling 2003). During the high water periods (when *Congeria* colonies are submerged) metabolic products of *Congeria* (urine, faeces) may alter the microenvironment around them, which may affect the composition of microorganisms, but it is expected that this impact will diminish in periods of drought as *Congeria* is in a dormant stage. The most distinctive common trait of cave protists and *Congeria* are habitats they share. For this reason the focus of this paper will be on testate amoebae assemblages present in cave habitats with *Congeria*, as opposed to other caves in the Lika region.

Existing data on cave protists in general are very limited. Caves are inhabited by mostly cosmopolitan protists described from epigean habitats (Gittleson and Hoover 1969, 1970; Golemansky and Bonnet 1994). Nevertheless, several species and forms have been described from caves (Chibisova 1967; Delhez and Chardez 1970; Walochnik and Mulec 2009; Baković et al. 2019) and cave entrances (Main 2003; Bhatt and Karthick 2020; Soler-Zamora et al. 2021). Protists are the trophic link between metazoans and prokaryotic organisms (archaea and bacteria), and thus they are important members of cave ecosystems. Considering their small size, short life cycle, quick response to environmental changes, numerous communities and diverse ecological niches they fill (Hausmann et al. 2003), protists are very compatible as indicator organisms (Foissner and Berger 1996; Bellinger and Siegee 2015). Till now, only ciliates have been investigated in the context of environmental indication in caves by Italian researchers (Coppellotti and Guidolin 1999). Baković et al. (2022a) showed that protist assemblages can be used for identification of habitat diversity in caves. Among protist from caves, great potential for researching cave ecology can be attributed to testate amoebae. Testate amoebae are very diverse in caves in comparison with other protists (Baković et al. 2019) and the species composition is determined by the specific habitats (Golemansky and Bonnet 1994) and cave heterogeneity (Mazei

et al. 2012). They have been recognized and tested as valuable indicators of environmental changes in many surface habitats such as peatlands, soil and aquatic habitats (e.g. Bobrov 2005; Qin et al. 2016; Carballeira and Pontevedra-Pombal 2021). Data on protists in the wider area of the Lika region are scarce. The only data on testate amoebae including caves from this research is that the Čakovac cave, the Horvatova cave and the Pražina cave are inhabited by the recently described species *Centropyxis bipilata* (Baković et al. 2019).

The goal of this study was to identify the diversity of testate amoebae present in selected caves of the Lika region and to determine whether there are differences between testate amoebae assemblages from caves inhabited by the endemic bivalve *C. jalzici* and other caves not inhabited by this species. This research brings the first data on heterotrophic protists associated with *C. jalzici* and the description of a new testate amoeba species for science.

Research location and hydrological settings

All studied caves are presented in Table 1 with cave name and location, sampling date, sampled habitat of testate amoebae and sample designation.

Markov ponor, Dankov ponor and Dražice ponor are sinkhole type caves formed in deposits of Jurasic limestones and dolomites ($J_3^{1,2}$, J_2, J_1^4). They are located on the NE edge of the Lipovo polje (karst field) (elevation approx. 451 m m.a.s.l.) (Fig. 1). These caves are natural sinkholes of the Lika river, but the flooding regime is modified after the building of two artificial dams (the Selište and the dam of the Senj Hydro Power Plant). Water is released in sinkholes just during very high waters. Markov ponor is very complex, multilevel objects, while Dankov ponor and Dražice ponor are less complex objects. Pražina pećina, Buklina, Pećina u Čakovcu and Horvatova špilja are formed in deposits of Eocen-Oligocen breccias-conglomerates (E, Ol) in the elevated area of Mlakva and Mlakvina greda (elevation 521–595m m.a.s.l.), while Samograd is formed in limestone deposits of the Upper Cretaceous, Cenoman and Turone in the Grabovača area (670 m m.a.s.l.) (Fig. 1). Horvatova špilja and Pećina u Čakovcu are multichannel caves. The Pražina pećina and Buklina caves are of a simple morphology. In the lower levels of Pećina u Čakovcu a cave lake is present. Horvatova špilja is located in the artificial dam of the Senj Hydro Power Plant and it is partially and occasionally flooded. In Pražina pećina and Buklina only seeping and dripping water is present. Total cave channel lengths are: Dankov ponor=250 m, Dražice ponor=approx. 140 m, Markov ponor=2557 m, Pražina pećina=27 m, Buklina=20 m, Pećina u Čakovcu=267 m, Samograd=345 m and Horvatova špilja=500 m. In the wider area of these caves building of new hydropower accumulation is planned in the future. It will completely flood the Pećina u Čakovcu and further modify the hydrological regime of Markov ponor, Dankov ponor and Dražice ponor by decreasing flooding frequency and quantities of water flooding these caves (Stroj 2010; Elektroprojekt Ltd. 2017).

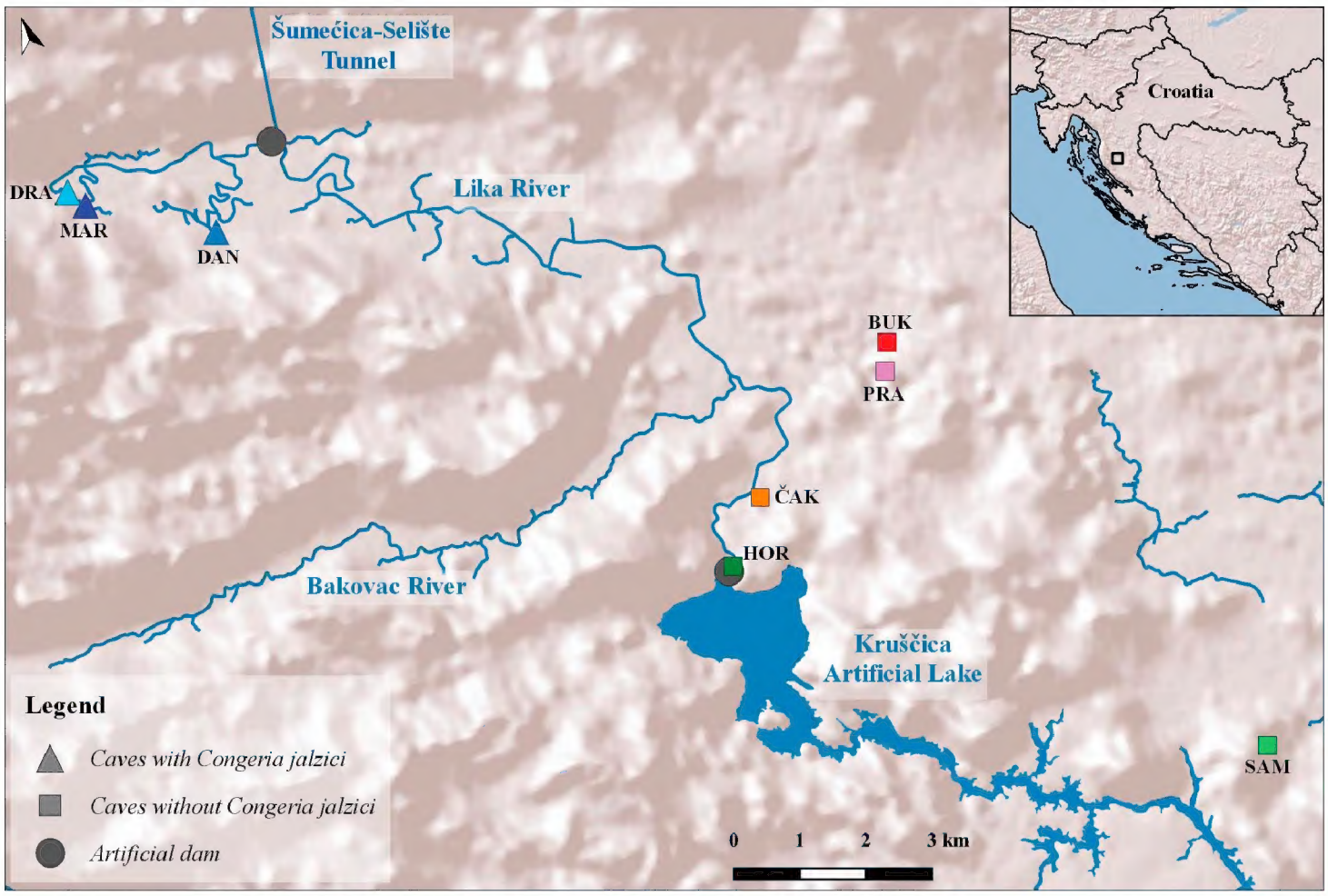

Figure 1. Research location and hydrographic map showing the main permanent and temporary water bodies and the hydrotechnical tunnel (DAN – Dankov ponor, DRA – Dražice ponor, MAR – Markov ponor, BUK – Buklina, PRA – Pražina pećina, ČAK – Pećina u Čakovcu, HOR – Horvatova pećina, SAM - Samograd).

Methodology

Description of studied habitats

Caves with *Congeria jalzici* (Markov ponor, Dankov ponor, Dražice ponor) are seasonally completely or partially flooded by the Lika river that sinks into these caves. Thus, habitats inside caves are transforming depending on the hydrological regime. These caves were studied during periods of low waters, when the caves were accessible to speleobiologists. Studied habitats were: a small cave lake with submerged colonies of *C. jalzici*, cave walls with attached individuals of *C. jalzici*, cave walls without individuals of *C. jalzici* and small cave pools unrelated to *C. jalzici*. The water present in a small cave lake with submerged colonies of *C. jalzici* is a remnant of water imported to the cave by the Lika river during the flood events. Organic debris and small waste (plastic etc.) fragments were present on the surface of the lake. The water level in these lakes gradually drops during the dry season, leaving the colonies of *C. jalzici* out of the water. Habitats, with attached *C. jalzici*, outside the water column are transitional habitats (hygropetric) and wet walls. Transitional habitats (hygropetric) are very thin and consist of slowly seeping water that flows over the surface of the cave wall and attached individuals of *C. jalzici*. Although there is no visible seeping water on the wet

walls, they are still permanently wet due to the high air humidity inside the cave. There are also several locations with small pools in the caves. The distinction between transitional habitats and wet walls is not always clear. The investigated sinter and clay pools in these caves are relatively small (a few centimeters wide and deep).

The investigated habitats in the caves without *C. jalzici* (Pražina pećina, Buklina, Pećina u Čakovcu, Horvatova špilja and Samograd) were not flooded by exogenous rivers. These researched habitats depended on seeping and dripping water. In most habitats of these caves traces of old bats guano are present, which is detectable in microscopical samples by the presence of small quantities of insects leftovers. Only in samples BUK_th and SAM_sp significant quantities of bats guano were found, while sample HOR_bg consisted of bats guano deposits (Table 1). Transitional habitats (hygropetric) in these caves were characterized by a very thin layer of seeping water that flows on the surface of the cave walls. The sinter pools in Pražina pećina and Samograd were 30 cm wide and up to 15 cm deep, while the sinter pools in Pećina u Čakovcu were several cm wide and deep.

Table 1. Collected samples.

No.	Cave name and location	Sampling date	Sampled habitat of testate amoebae	Sample designation
Caves with Congeria jalzici				
1	Dankov ponor, Lipovo polje, CRO	08/2016*	Transitional habitat with *C. jalzici*	DAN_th
2	Dankov ponor, Lipovo polje, CRO	08/2016	*Ex situ* swabs of shells of living *C. jalzici*	DAN_esw
3	Dražice ponor, Lipovo polje, CRO	08/2016*	Transitional habitat with *C. jalzici*	DRA_th
4	Dražice ponor, Lipovo polje, CRO	11/2018	Transitional habitat	DRA_th2
5	Dražice ponor, Lipovo polje, CRO	11/2018	Transitional habitat	DRA_th3
6	Dražice ponor, Lipovo polje, CRO	11/2018	*In situ* swabs of shells of living *C. jalzici*	DRA_isw
7	Dražice ponor, Lipovo polje, CRO	11/2018	*Ex situ* swabs of shells of living *C. jalzici*	DRA_esw
8	Markov ponor, Lipovo polje, CRO	09/2016*	Transitional habitat with *C. jalzici*	MAR_th1
9	Markov ponor, Lipovo polje, CRO	09/2016*	Transitional habitat with *C. jalzici*	MAR_th2
10	Markov ponor, Lipovo polje, CRO	10/2016	Plankton from cave lake (65 µm mesh)	MAR_pla1
11	Markov ponor, Lipovo polje, CRO	10/2016	Plankton from cave lake (120 µm mesh)	MAR_pla2
12	Markov ponor, Lipovo polje, CRO	10/2016	Floating debris from cave lake	MAR_deb
13	Markov ponor, Lipovo polje, CRO	11/2021	Transitional habitat	MAR_th1
14	Markov ponor, Lipovo polje, CRO	11/2021	Transitional habitat	MAR_th2
15	Markov ponor, Lipovo polje, CRO	11/2021	Sinter pool filled with water	MAR_sin
16	Markov ponor, Lipovo polje, CRO	11/2021	Clay pool	MAR_clp
Caves without Congeria jalzici				
17	Buklina (syn. Špilja u Poljani), Poljana, CRO	08/2016*	Transitional habitat	BUK_th
18	Horvatova špilja, HPP Sklope, Mlakva, CRO	08/2016*	Bats guano deposits	HOR_bg
19	Horvatova špilja, HPP Sklope, Mlakva, CRO	08/2016*	Transitional habitat	HOR_th
20	Pećina u Čakovcu, Bobići, Mlakva, CRO	08/2016*	Transitional habitat	ČAK_th1
21	Pećina u Čakovcu, Bobići, Mlakva, CRO	08/2016*	Terrestrial sediment	ČAK_terr
22	Pećina u Čakovcu, Bobići, Mlakva, CRO	09/2016*	Transitional habitat	ČAK_th2
23	Pećina u Čakovcu, Bobići, Mlakva, CRO	09/2016*	Sinter pool filled with water	ČAK_sp
24	Pražina pećina, Poljana, Mlakva, CRO	08/2016*	Transitional habitat	PRA_th
25	Pražina pećina, Poljana, Mlakva, CRO	08/2016*	Sinter pool filled with water	PRA_sp
26	Samograd, Grabovača, Perušić, CRO	10/2016*	Transitional habitat	SAM_th
27	Samograd, Grabovača, Perušić, CRO	10/2016*	Sinter pool filled with water	SAM_sp

Samples marked with asterisk (*) were used for a descriptive statistics; other samples were used exclusively for the description of alpha diversity of testate amoebae.

Sampling

The samples from Markov ponor (MAR_th1, MAR_th2), Dankov ponor (DAN_th) and Dražice ponor (DRA_th) (Table 1) were collected by gently scrubbing with a plastic brush the surfaces of shells of living *Congeria jalzici* and the transitional habitats on which they were attached. The brush was then washed in a plastic container filled with 40 ml of tap water. The process was repeated till the water became muddied. Selected samples from Markov ponor (MAR_th1, MAR_th2, MAR_sin, MAR_clp) and Dražice ponor (DRA_th2, DRA_th3), and all samples from the caves Pražina pećina, Pećina u Čakovcu, Horvatova špilja and Samograd (Table 1) were collected by using the methodology presented by Baković et al. (2019).

Plankton samples from Markov ponor (MAR_pla1, MAR_pla2) were collected using plankton nets (65 μm and 120 μm mesh size) and placed into plastic containers. Floating debris from the cave lake (MAR_deb) was handpicked and placed into a plastic container filled with lake water (Table 1).

Samples of protists living *in situ* on the outer shells of *C. jalzici* (DRA_isw) were collected by gently scrubbing with a plastic brush exclusively on the surfaces of shells of living *C. jalzici* on transitional habitats. The brush wa then washed in a plastic container filled with 40 ml of tap water. The process was repeated until the water became cloudy. *Ex situ* samples of protists living on the outer shells of *C. jalzici* (DRA_esw, DAN_esw) were scrubbed gently with a microscope cover slip (Table 1). Scrubbed material was transferred directly to a microscope slide. One drop of water was added to the microscope slide before the examination.

Transport to the lab, maintaining the temperature, examination

Samples were transported to the laboratory within several hours after the sampling and stored at a temperature of 4–8 °C and analyzed within 48h from the time of the collection. Exceptions were selected samples from Markov ponor (MAR_pla1, MAR_pla2, MAR_deb) (Table 1) which were stored at room temperature and examined within a 72 hour time period from the time of collection.

Triplets of 0.2 ml from each sample (total of 0.6 ml) were examined using a Carl Zeiss Axiostar microscope with 100, 400 and 1000 times magnification. For additional species examination a Zeiss Axioscop 40 FL and an Olympus BX51 microscope with Phase Contrast and Differential Interference Contrast (DIC) were used. A Nikon Diaphot inverted microscope was used for examining samples and isolating specimens. Adobe Photoshop and ToupView software were used for image processing and measurements. All testate amoebae were identified to the lowest possible level. Species were identified by using the following literature to begin with: Golemansky and Todorov (2004), Mazei and Tsyganov (2006), Nicholls (2005), Ogden and Hedley (1980), Siemensma (2021) and Todorov and Bankov (2019). An abundance of testate amoebae is noted only for samples no. 1–2, 5–6, 14–24. Counted were all individuals in the examined volume (0.6 ml). Based on the number obtained, it was estimated (mathematical calculation) how many individuals would be in 1 ml. Since literature data on testate amoebae abundance in caves

are lacking, available data from this research were used to determine ranges (low/high abundance). Samples used only for the studding testate amoebae diversity were intensively examined in search of testate amoebae tests (> than 0.6 ml of sample was examined).

Data analyses

Descriptive statistics (Bray-Curtis similarity, Shannon diversity index and Pielou's evenness index) was done in Primer 6 (PRIMER-e Ltd) on selected samples collected in 2016 (samples no. 1–2, 5–6, 14–24) which represented comparable data. Other samples (3, 4, 7, 8, 9, 10, 11, 12, 13) were used only for the contribution to knowledge on testate amoebae diversity.

Results

Taxa diversity

Testate amoebae were found in 23 samples (85.1%). Exceptions were the samples of the cave lake plankton (MAR_pla1, MAR_pla2) and in *ex situ* swabs from the shells of *C. jalzici* (DAN_esw, DRA_esw) that did not reveal any testate amoebae. Over forty testate amoebae taxa were distinguished (Table 2, Plates 1–3). Among them, 33 taxa have been identified to the species level, while others have been identified to the genus level. Several taxa were identified just as testate amoebae due to the small number of observed individuals that prevented accurate identification. Particularly diverse were taxa of the genera *Centropyxis* and *Difflugia*. The highest diversity of taxa (27) was found in Markov ponor. Among the taxa found during this research, one new species for science was identified and further described in this research – *Psammonobiotus dinarica* sp. nov.

Taxonomic description

Phylum: Cercozoa Cavalier-Smith 1998, emend. Adl et al. 2005; emend. Cavalier-Smith 2018
Class: Thecofilosea Cavalier-Smith 2003, emend. Cavalier-Smith 2011
Family: Incertae sedis Psammonobiotidae Golemansky 1974, emend. Meisterfeld 2002
Genus *Psammonobiotus* Golemansky, 1967

***Psammonobiotus dinarica* Baković & Siemensma, sp. nov.**
https://zoobank.org/89057128-7C93-434D-BA9E-A0CF8F4E7FAB
Plates 2F, 3A–E

Diagnosis. Shell is bilaterally symmetrical, in dorsal and ventral views spherical to ovoid in outline and in lateral view compressed with a length/height ratio of about 2.3. A funnel-shaped collar extends from a kidney-shaped oral aperture. In lateral view,

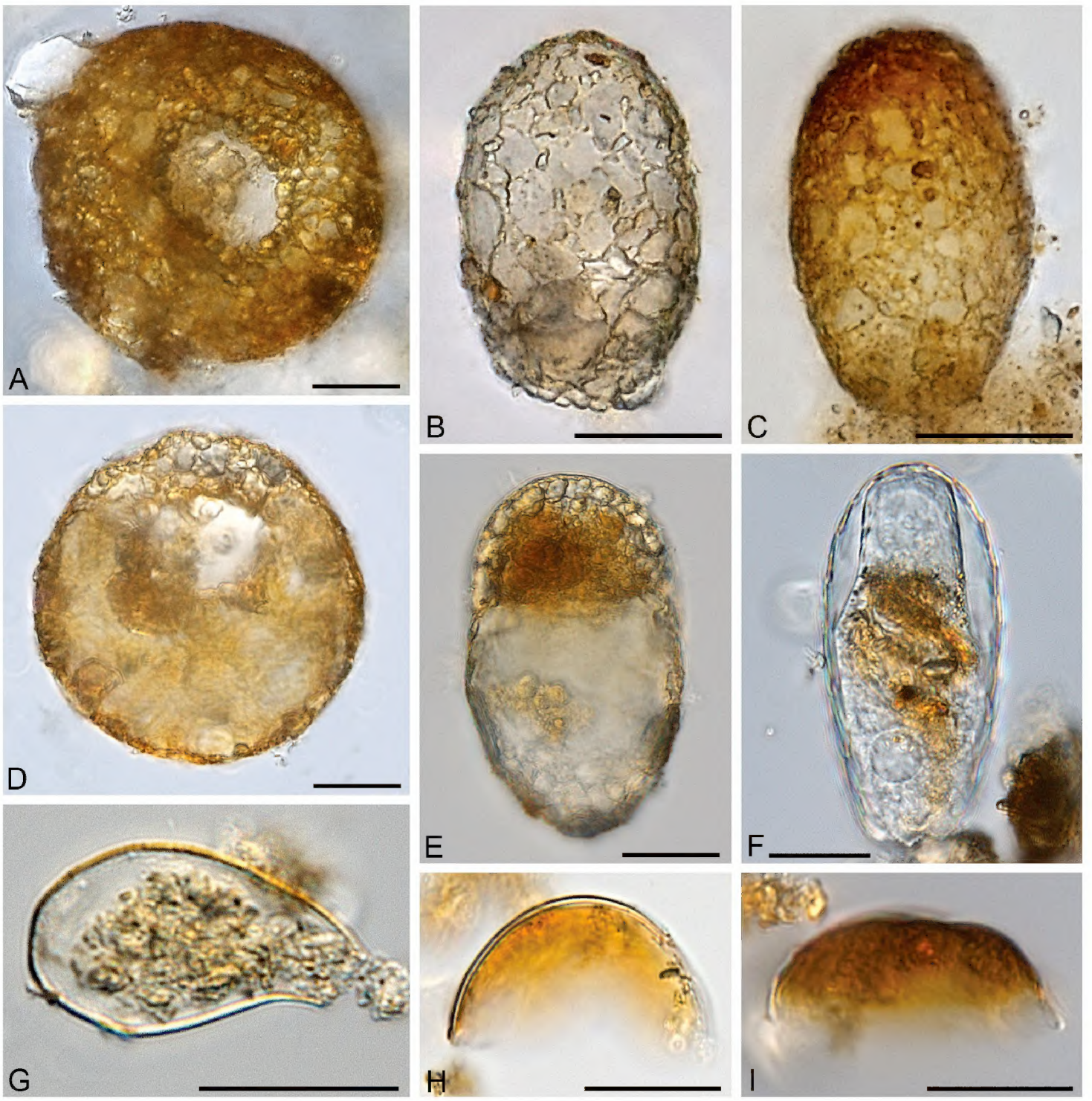

Plate 1. A *Cyclopyxis kahli* **B** *Difflugia* cf *pristis* **C** *Pseudodifflugia gracilis* **D** *Centropyxis aerophila* **E** *Centropyxis constricta* **F** *Euglypha tuberculata* **G** *Cyphoderia laevis* **H, I** *Microchlamys patella*. Scale bars: 20 μm.

the angle of the plane of this pseudostome collar is usually zero degrees, but can sometimes be as high as 33°. The translucent and fragile organic shell is covered with small irregularly-shaped thin and flat quartz particles. Larger particles are located on the dorsal and distal part of the shell and smaller particles on the ventral side. The rim of the collar is covered with relatively large flat particles. The organic matrix is colorless to dark brown. Length including the collar 45–54 μm; main body width 26–30 μm, height 17–30 μm; collar 20–29 μm across (n=6).

Etymology. The specific name refers to the area where the species was found, the Dinarides or Dinaric Alps, Latin: *dinarica*, a mountain range in, among others, Croatia and Bosnia and Herzegovina.

Type material. Three slides, one with the ***holotype*** and two with ***paratypes***, were mounted in HYDRO-Matrix on glass slides and deposited in the collection of the

Croatian Biospeleological Society under accession numbers TAM4 (holotype), TAM5 and TAM6 (paratypes).

Type locality. Croatia, Lika region, Lipovo polje, Dražice ponor, 44°46'20.2"N, 15°11'10.6"E, 10 November 2018, H. Bilandžija leg.

Differential diagnosis. There are several testate amoebae similar in shape and size to *P. dinarica*: *Centropyxis platystoma*, *Psammonobiotus communis*, *P. septentrialis* and *P. minutus*, some *Centropyxiella* and *Corythionella* species, and *Conicocassis pontigulasiformis*. *Centropyxis platystoma* was described by Penard in 1890, but in 1902 he considered this species identical to Leidy's *C. constricta* (Penard, 1890, 1902). Since he originally described it as *Difflugia platystoma*, the shell must have looked to him as that of a *Difflugia*, with a dense covering of quartz particles. Penard described the shell as "pierreuse", stony. This is different from *P. dinarica*, where the shell is covered with tiny flat particles. In the original description, Penard showed a drawing of the visor with a strongly inwardly curved edge, in contrast to the edge of the collar of *P. dinarica* which is not curved. *P. dinarica* can be distinguished from *P. septentrialis* and *P. minutus* by its larger size, 45–54 μm long versus 10–12 μm and 23–30 μm, respectively (Golemansky 1970; Chardez 1977a). The size is about the same as *P. communis*, 45–54 μm (Golemansky 1967) versus 33–52 μm, but *P. dinarica* can be distinguished from the latter by the kidney-shaped aperture. *P. dinarica* also bears some resemblance to *Conicocassis pontigulasiformis*, but this species is much larger, 82–90 μm, and the test wall has a much more pronounced granular organic cement and a circular aperture (Nasser and Patterson 2015). *P. dinarica* can be distinguished from *Centropyxiella arenaria* by its kidney-shaped aperture, and from *C. elegans* and *C. lucida* by its smaller size, length 45–54 μm versus 70–80 μm and 70–81 μm, respectively (Golemansky and Todorov 2007). It can be distinguished from similarly shaped *Corythionella* species (Golemansky 1970b) by the presence of small elliptical or oval idiosomes in the latter genus.

Biogeography and habitats. *Psammonobiotus dinarica* was found in the Dinaric karst of Croatia and in Bosnia and Herzegovina, in the caves Dražice ponor, Markov ponor (both Lipovo polje, CRO) (Fig. 1, Table 1), Jopićeva cave-Bent system (Krnjak, CRO) and Matešićeva-Popovačka cave system (Slunj, CRO) (Baković N., unpublished data). One empty shell was found in a small karst spring near the Jopićeva cave-Bent system (Krnjak, CRO) (Baković N., unpublished data). This species was also reported in the Velika Bukovačka and the Listvača cave (both in Bosnia and Herzegovina) (Baković et al. 2019). The altitude of these localities ranges from 210 to 950 m.a.s.l. The species inhabits surface sediments of permanent cave streams (dominant), sediments of small cave standing waters (sinter pools and clay pools) and transitional habitats (hygropetric). Only one observation of *P. dinarica* on the shell of a living *Congeria jalzici* was noted.

The following ranges of physio-chemical parameters were present in the habitat of this species: water temperature 5.4–10.7 °C, pH 7.62–8.11, conductivity 161–338 μS/cm. It was only occasionally present in all investigated habitats, but always at low densities (up to 3.3 ind. in 1 ml of aquatic sediment).

Table 2. Taxa diversity.

	Taxa	Location							
		DAN	DRA	MAR	BUK	ČAK	HOR	PRA	SAM
1	*Centropyxis aculeata* Ehrenberg, 1838			+					
2	*Centropyxis aerophila* Deflandre, 1929		+	+		+			+
3	*Centropyxis constricta* (Ehrenberg, 1841) Penard, 1890			+					
4	*Centropyxis bipilata* Baković, Siemensma et. al, 2019					+	+	+	
5	*Centropyxis elongata* (Penard) Thomas, 1959			+			+		
6	*Centropyxis laevigata* Penard, 1890		+			+			
7	*Centropyxis plagiostoma* Bonnet et Thomas, 1955			+					
8	*Cryptodifflugia oviformis* Penard, 1902			+	+		+		+
9	*Cyclopyxis eurystoma* Deflandre, 1929		+	+		+	+		
10	*Cyclopyxis kahli* Deflandre, 1929			+					
11	*Cyclopyxis* sp 1					+			
12	*Cyclopyxis* sp 2							+	
13	*Cyphoderia ampulla* (Ehrenberg, 1840) Leidy, 1878			+				+	
14	*Cyphoderia laevis* Penard, 1902			+					
15	*Cyphoderia* sp.			+					
16	*Difflugia lithophila* Penard, 1902		+					+	
17	*Difflugia lucida* Penard 1890		+						
18	*Difflugia* cf. *pristis* Penard, 1902		+	+					
19	*Difflugia oblonga* Ehrenberg, 1838			+					
20	*Difflugia penardi* Hopkinson, 1909		+						
21	*Difflugia pulex* Penard, 1890		+	+					
22	*Difflugia* sp 1		+	+					
23	*Difflugia* sp 2					+		+	
24	*Difflugia* sp 3							+	
25	*Euglypha* sp.			+					+
26	*Euglypha laevis* Perty, 1849			+			+		
27	*Euglypha rotunda* Wailes & Penard, 1911			+	+		+	+	
28	*Euglypha tuberculata* Dujardin, 1841			+					
29	*Heleopera petricola* Leidy, 1879		+					+	
30	*Heleopera* sp.						+		
31	*Heleopera sylvatica* Penard, 1890					+			
32	*Microchlamys patella* (Claparède et Lachmann, 1859) Cockerell, 1911	+	+	+		+			
33	*Paraquadrula irregularis* (Archer, 1877) Deflandre, 1932					+	+		
34	*Parmulina* sp.			+					
35	*Phryganella paradoxa* Penard 1902		+						
36	*Plagiopyxis declivis* Bonnet, 1955			+					
37	*Psammonobiotus dinarica* sp. nov.		+	+					
38	cf *Psammonobiotus linearis* Golemansky, 1971		+						
39	*Pseudodifflugia gracilis* Schlumberger, 1845		+	+					
40	Testacea sp 1		+						
41	Testacea sp 2		+			+			
42	Testacea sp 3		+						
43	Testacea sp 4					+			
44	*Tracheleuglypha dentata* (Vejdovsky, 1882) Deflandre, 1928			+	+		+		+
45	*Trinema enchelys* Ehrenberg, 1838			+					
46	*Trinema lineare* Penard, 1890		+	+		+	+	+	+
	Total	1	19	27	3	12	10	9	5

Locations: DAN – Dankov ponor, DRA – Dražice ponor, MAR – Markov ponor, BUK – Buklina, PRA – Pražina pećina, ČAK – Pećina u Čakovcu, HOR – Horvatova pećina, SAM - Samograd.

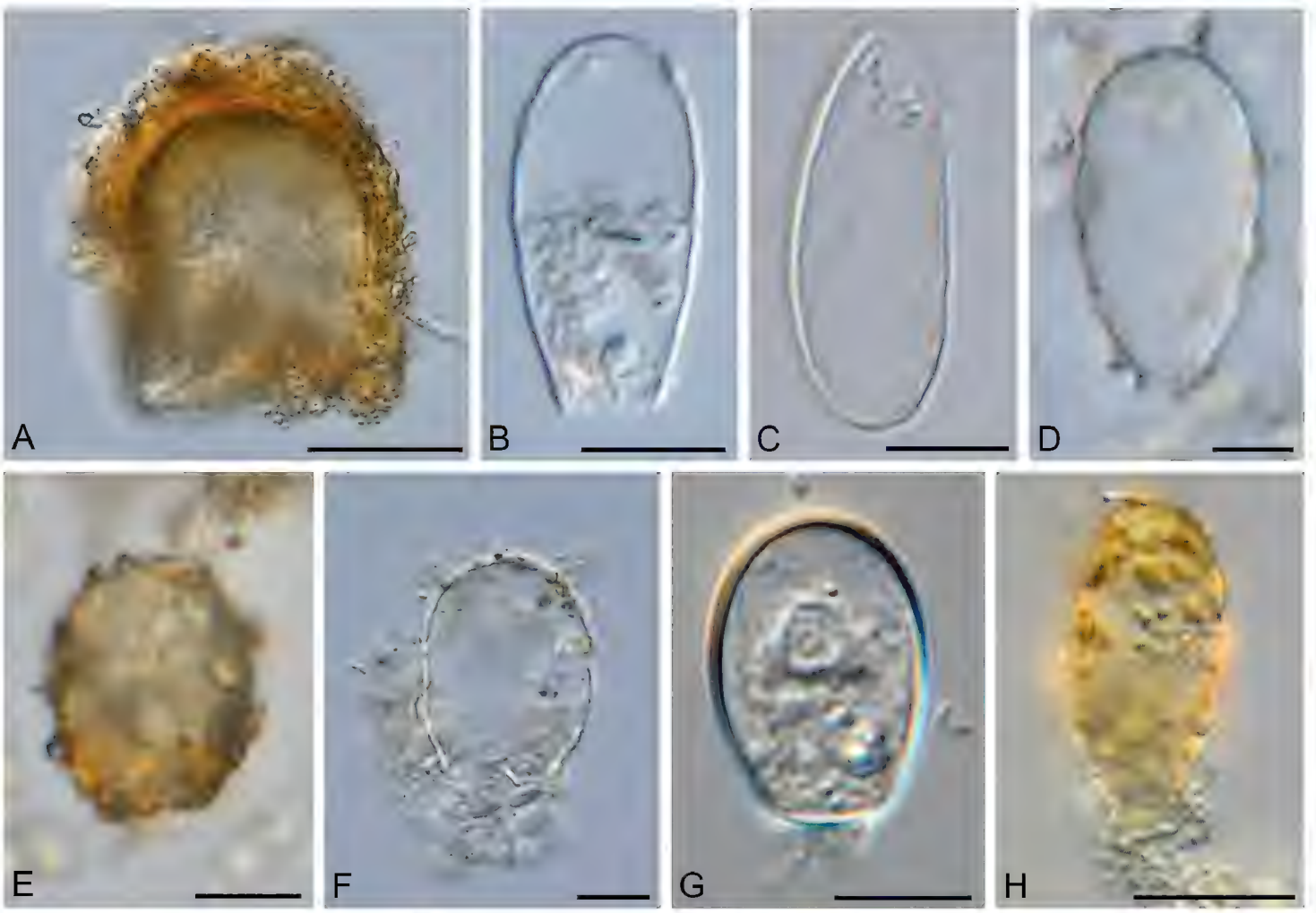

Plate 2. A *Parmulina* sp. **B** *Euglypha laevis* **C** *Trinema lineare* **D** *Tracheleuglypha dentata* **E** *Phryganella paradoxa* **F** *Psammonobiotus dinarica* sp. nov. **G** *Cryptodifflugia oviformis* **H** Testacea sp 1 **I** Testacea sp. 2 **J** Testacea sp. 3 (stacked image) **K** *Centropyxis laevigata*. Scale bars: 20 μm (**A, I–K**); 10 μm (**B–H**).

Remarks. The genus *Psammonobiotus* contains nine beach sand-dwelling species, six recorded only from marine and brackish waters (Golemansky and Todorov 2004), one exclusively from freshwater (*P. dziwnowi*) (Nicholls 2005) and two also from marine and freshwater bodies (*P. communis* and *P. linearis*) (Golemansky and Todorov 2004). *P. dinarica* is the second *Psammonobiotus* species to be reported exclusively from a freshwater biotope. All species known to date have been collected from the psammon from beaches of marine water bodies and some large freshwater lakes (Golemansky and Todorov 2005). The presence of *P. dinarica* in a (freshwater) cave system can therefore be called surprising. However, it is not the first *Psammonobiotus* species to have been found in another ecosystem. The website arcella.nl reports the presence of *Psammonobiotus*

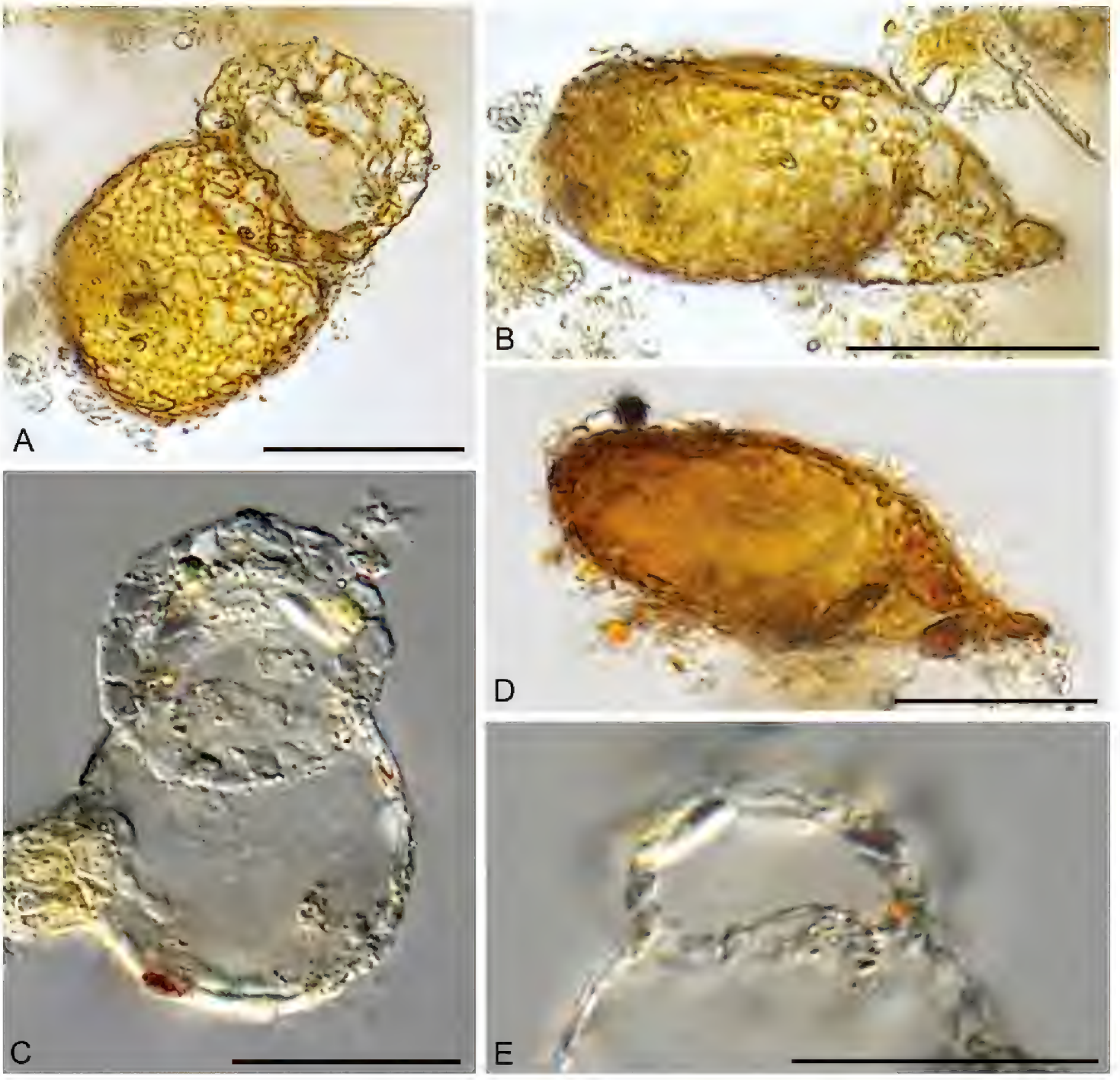

Plate 3. *Psammonobiotus dinarica* **A** shell in ventral view (type specimen) **B** shell in lateral view (type specimen) **C** shell in ventral view **D** shell in lateral view **E** kidney-shaped aperture. Scale bars: 20 μm. **A–D** stacked photomicrographs.

species in a relatively small lake, in organic sediment of a small shallow freshwater ditch, in organic sediment of an isolated pond and in the soil of a peat bog, all in the Netherlands (Siemensma 2021). It shows that our knowledge about the presence and distribution of *Psammonobiotus* species in freshwater and soil biotopes is still very limited.

Regarding the presence of light, all species of the genus *Psammonobiotus* primarily inhabit aphotic biotopes – cave sediments (*P. dinarica*) and interstitial sand habitats (all other species) (Golemansky and Todorov 2005).

Although living specimens of *P. dinarica* have been observed, pseudopodia could never be observed as these specimens were always firmly attached to sediment particles. The brown color, if present, disappears rapidly when the shell is embedded in HYDRO-Matrix.

Testate amoebae assemblages

Comparison of taxa diversity (Fig. 2) showed that most diverse were the sample of bats guano (HOR_bg) and one sample from a sinter pool (ČAK_sp) where guano was not present. Diversity and abundance of testate amoebae in samples from caves inhabited by *C. jalzici* did not show any specific deviation – they vary from low to medium diversity and abundance of taxa.

The highest taxa abundance (Fig. 2) was detected in the sample of bats guano HOR_bg (74 ind. in 1 ml) and two other samples rich in bats guano: BUK_th (55 in. in 1 ml) and SAM_sp (41 ind. in 1 ml). Despite the high taxa diversity, samples from Markov ponor showed relatively a low abundance of taxa (<25 ind. in 1 ml). The lowest abundance was detected in samples of ČAK_th2 (6 ind. in 1 ml), DAN_th, DRA_th, ČAK_sp, HOR_th (all 12 ind. in 1ml) and PRA_th (13 ind. in 1 ml).

Regarding the abundance of individual testate amoebae in caves inhabited by *C. jalzici*, the species that can be highlighted is *Microchlamys patella*. It was present in all the samples from these caves and it was relatively abundant - reaching 5–10 ind. in 1 ml (average 7.08 ind. in 1 ml). In comparison, the abundance of six other testate amoebae present in these caves was 0–3.3 ind. in 1 ml (average 0.41–1.25 ind. in 1 ml). Also, they were not present in all the samples.

Shannon diversity and Pielou's evenness (Fig. 3) did not show specific difference between caves inhabited with *C. jalzici* and other caves. The differences rather varied depending on the individual samples. Highest Shannon index values were in samples MAR_th1 (3.51), ČAK_sp (3.08) and HOR_bg (3.07). The Pielou's evenness was higher in samples ČAK_sp (0.97), ČAK_th2 and DAN_th (both 0.96). The Shannon diversity and Pielou's evenness for the samples DAN_th and SAM_th were zero due to the only one species present in the samples.

The differences between caves inhabited with *C. jalzici* and other caves were well visible in Bray-Curtis similarity dendrogram based on diversity and abundance of the testate amoebae (Fig. 4). Three main branches are distinguished: samples from caves inhabited with *C. jalzici* (MAR_th1, MAR_th2, DAN_th, DRA_th), samples with significant presence of bats guano (BUK_th, HOR_bg, SAM_sp) and other samples from caves uninhabited by *C. jalzici* (all other). Among third branch it could be emphasized higher similarity of sample of terrestrial cave habitat (ČAK_terr) with transitional habitats (ČAK_th1, HOR_th, SAM_th) in contrast to branches containing sinter pools (small aquatic habitats).

Discussion

This research contributes with the first data on testate amoebae inhabiting three caves with the endemic and endangered cave bivalve *Congeria jalzici*, as well as with data from five other caves in the Lika region. Species found in caves in this study are mostly eurybiotic species also present in surface habitats, which is in accord with literature data (Mazei et al. 2012). Among the recorded species is one known only

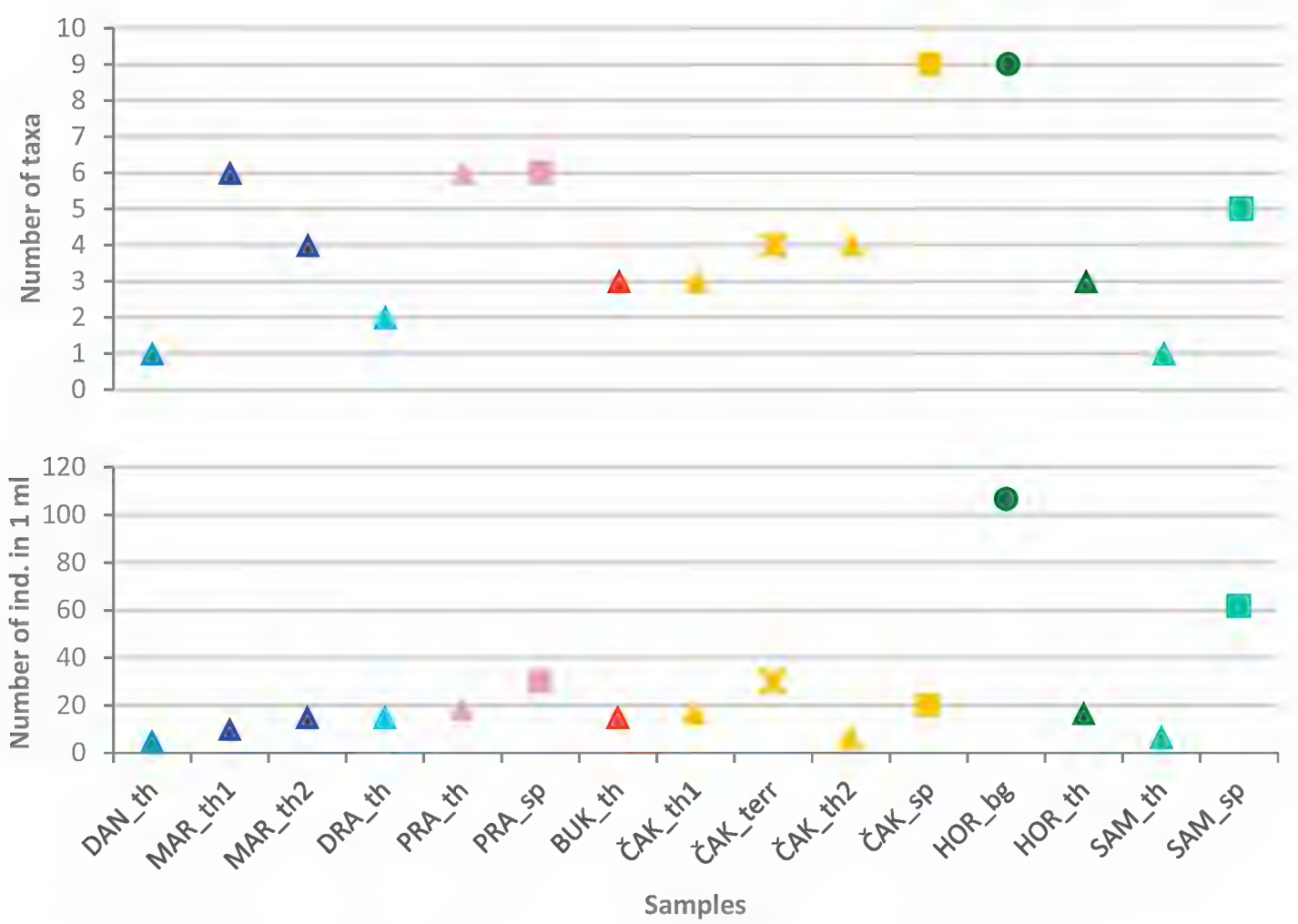

Figure 2. Diversity and abundance of testate amoebae (marker shape: triangle – transitional habitats, asterisk – terrestrial habitats, circle – bats guano deposits, square – sinter pools; marker color: represent different caves).

from caves (*Centropyxis bipilata*) (Baković et al. 2019, 2022a) and one new to science (*Psammonobiotus dinarica* sp. nov.). Species and forms known exclusively from caves (Mazei et al. 2012; Baković et al. 2019; Soler-Zamora et al. 2021), imply that caves may harbor even more species new to science that have been confused with eurybiotic species or have been overlooked (especially in understudied regions).

Despite the fact that an empty shell of *P. dinarica* sp. nov. has been found once in a small karst spring near Jopićeva cave-Bent system (unpublished data, N. Baković), it does not prove beyond doubt that this species also occurs in surface waters, as it could easily be washed away from the subterranean habitats. The full extent of its habitats requires further study. As one individual of *P. dinarica* was found on the shell of a live bivalve *C. jalzici*, this raised the question of whether they were somehow connected. There are many records of epibiotic ciliates associated with cave animals (Matjašič 1962; Dovgal and Vargovitsh 2010) together with some protists that are animal parasites (Gittleson and Hoover 1969), but there is currently no data to support that *P. dinarica* is in some way related to *C. jalzici* in terms of interspecies relations.

The comparison of the testate amoebae diversity in the researched caves was limited due to the small number of samples examined and different time points in which they were collected. Still results from 27 testate amoebae from Markov ponor (from only nine samples) imply that their diversity in individual caves may be even higher than previously reported from Vjetrenica cave in Bosnia and Herzegovina (25 spe-

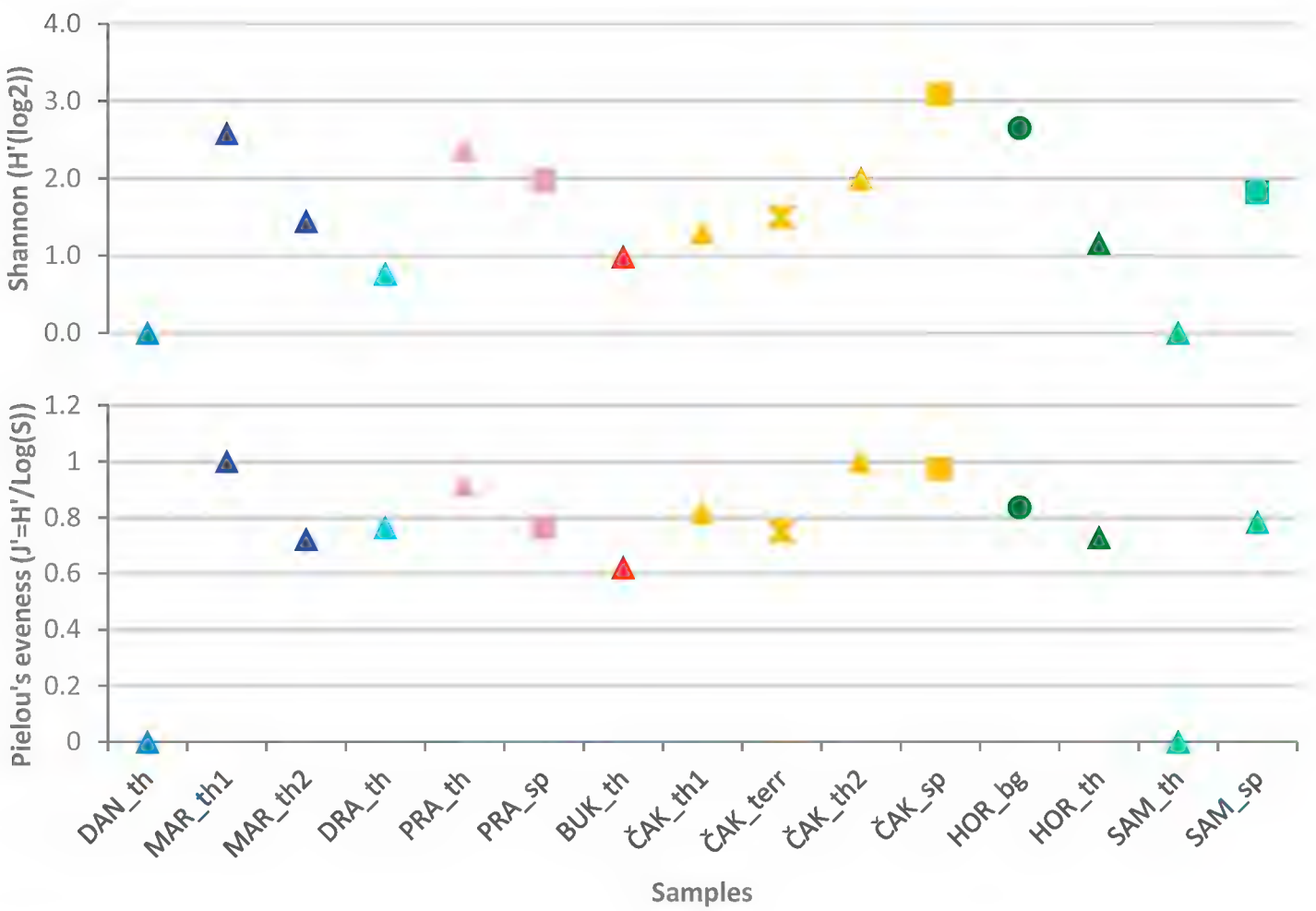

Figure 3. Diversity indices based on testate amoebae assemblages (marker shape: triangle – transitional habitats, asterisk – terrestrial habitats, circle – bats guano deposits, square – sinter pools; marker color: represent different caves).

cies) (Ozimec et al. 2021) and Covo della Guera in Italy (21 species) (Mazei et al. 2012). Nevertheless, data from Baradla Cave (Hungary), where 45 species were found by several researchers (Bereczky 1970), imply that the diversity of testate amoebae in caves could be much higher. The high diversity of Markov ponor may be partly due to flooding by the Lika river. These events could import some species from the surface streams into cave. The comparative study of testate amoebae inside the caves and the riverbeds of sinking streams is completely missing in the literature data. The results on taxa diversity, abundance (Fig. 2) and diversity indices (Fig. 3) from this research were not inconclusive regarding the separation of caves inhabited by *Congeria jalzici* from other caves. The differences between samples can be regarded as differences between habitats and the presence of bats guano that have already been noted by other authors (e.g. Golemansky and Bonnet 1994). When guano is present in caves, it has multiple effects. It increases the suplly of nutrients, which boosts the diversity and abundance of microorganisms, lowers the pH of habitats (Mulec et al. 2015) and provides a more heterogeneous habitat for microorganisms (Baković et al. 2022a).

The main result of this research is the distinction between samples from caves inhabited by cave bivalve *C. jalzici* and other investigated caves based on Bray-Curtis similarity (Fig. 4). The caves inhabited by *C. jalzici* are seasonally completely or partially flooded by the exogenous Lika river (for several weeks or even months). These

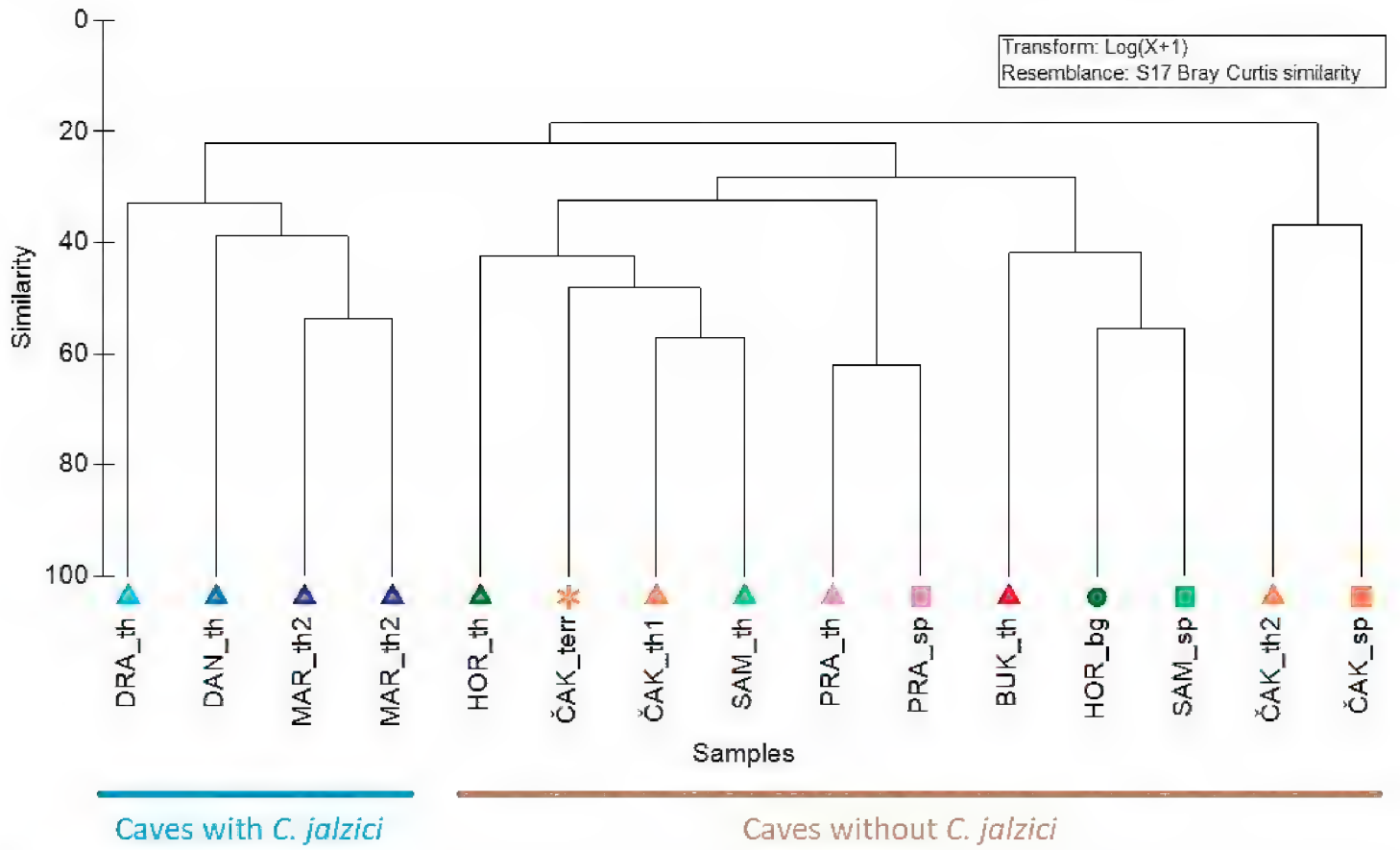

Figure 4. Bray-Curtis similarity dendrogram (marker shape: triangle – transitional habitats, asterisk – terrestrial habitats, circle – bats guano deposits, square – sinter pools; marker color: represent different caves).

flood events mark nutrient-rich periods in these caves as the Lika river imports organic matter into cave habitats that are usually poor in organic matter (Simon et al. 2007). Even after the floodwaters are drained, large amounts of organic material remain in the cave (e.g. water left in cave pools, wood debris). The second way of introduction of surface waters into the wider area of these caves is by water runoff (water losses) through the Lika and Bakovac riverbeds and existing accumulation (artificial lake) Kruščica due to the karstification of this area (Pavičić 1997), a phenomenon that is very common in karst areas (Bonacci 1987; Bonacci and Rubinić 2009). Thus, the impact of surface waters of the Lika and Bakovac rivers together with the artificial lake Kruščica can also be present outside of the period of flooding.

During the dry period, the hygropetric in caves inhabited by *C. jalzici* visually resembles hygropetric in caves not subjected to flooding, as they are also characterized by slowly seeping and dripping water. But one key difference is that they are subjected to turbulent water movement during flood events that continuously wash these surfaces, and then, over the time of the water depletion, the suspended material is slowly depositing on the cave walls. Therefore, the results of this research confirmed that the hydrological conditions create a distinct habitat for the testate amoebae in caves that are subject to flooding. The impact of freshwaters on these habitats is also confirmed by the relatively high abundance of *Microchlamys patella.* It is an eurybiotic species, but is more dominant in the freshwater habitats (e.g. Yang et al. 2005; Vincke et al. 2006; Davidova and Vasilev 2012) in contrast to terrestrial and transitional habitats.

The influence of bat guano was also detected in results of Bray-Curtis similarity (Fig. 4), as the samples richest in bat guano (BUK_th, HOR_bg, SAM_sp) are assorted as separate branches, implying a specific response of testate amoebae on increased energy input and increased habitat heterogeneity (Mulec et al. 2016; Baković et al. 2022a). Specific influence of bat guano was also noted by Golemansky and Bonnet (1994), who mentioned a dense population of testate amoebae in bat guano deposits.

Conclusion

This study presents data on testate amoebae from eight caves of the Lika region in Croatia including the description of a new species for science, *Psammonobiotus dinarica* sp. nov. As heterotrophic protists in caves are scarcely researched, finding new species implies that caves could harbor more, still unknown, protists. The analyses from this study showed that testate amoebae assemblages differ in caves inhabited by *Congeria jalzici* in contrast to other caves studied. These differences can be attributed to the seasonal flooding that provides specific habitats for protists, as well as providing optimal conditions for the survival of endangered endemic *Congeria* species. This diversification needs to be further studied to better understand the conditions that shape specific protists assemblages. Except for the knowledge of cave testate amoebae, these cognitions could further enhance our understanding of the cave ecosystem and the conditions that sustain populations of *C. jalzici*.

Acknowledgements

We thank the following individuals that accompanied us in the field work: Damir Basara, Predrag Rade, Hrvoje Cvitanović, Gordan Polić and members of the Croatian Biospeleological Society and the Breganja Society. Special thanks to Vedran Sudar, Helena Bilandžija, Branko Jalžić and Damir Basara who provided us with several samples; Neven Matočec and Ivana Kušan for the provision of additional microscopy equipment for detail study of protists; Sandra Hudina for useful advices on statistics. Special thanks also to Korana Baković for her technical support during the field trips. This research was supported by the projects Biospeleological research in area of Lipovo polje (HEP, 2016), Dedicated institutional funding for science (2020/21) - Ministry of Science and Education, Republic of Croatia and non-project activities of the Society ADIPA (2021) and the Croatian Biospeleological Society (2018). The study was performed under permissions of the Croatian Ministry of Environmental Protection and Energy (Class: UP/1-612-07 /16-48/77; Ref. No.: 517-07-1-1-1-16-4, Zagreb, 10.05.2016; Class: UP/I-612-07/17-33/03, Ref. No.: 17-07-2-1-2-17-3, 08.03.2017) and the Ministry of Economy and Sustainable Development (Class: UP/I-612-07/21-48/170, Ref. No.: 517-10-1-1-21-3, 16.07.2021.).

Some of the results from this paper have been presented at the 3rd Symposium of Freshwater Biology, 15th February 2019 in Zagreb, Croatia.

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